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Award Number: W81XWH-11-1-0468

TITLE: Regulation and Function of TIFAB in Myelodysplastic Syndrome

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REPORT DATE: R } ^ A € 13

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188	
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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		8. PERFORMING ORGANIZATION REPORT NUMBER	
		10. SPONSOR/MONITOR'S ACRONYM(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
13. SUPPLEMENTARY NOTES			
14. ABSTRACT Myelodysplastic syndromes (MDS) are clonal bone marrow failure (BMF) disorders defined by blood cytopenias due to ineffective hematopoiesis, genomic instability, and a predisposition to acute myeloid leukemia (AML). The most commonly recurring genomic alteration in MDS is deletion of chromosome 5q (del(5q)). MDS patients with an isolated del(5q) presenting with anemia, neutropenia, and elevated platelets associated with dysplastic megakaryocytes are considered to have 5q- syndrome. The majority of MDS patients with del(5q) do not exhibit these particular symptoms and, instead, are referred to as "del(5q) MDS". We have recently identified miR-146a, which target the TRAF6 arm of the innate immune pathway, a gene within the deleted region in del(5q) MDS. We posit that multiple genes on chr 5q coordinate TRAF6 activation in del(5q) MDS. A search of annotated genes within or near the CDRs revealed a known inhibitor of TRAF6, TIFAB, on band q31.1. We hypothesize that deletion of TIFAB promotes activation of the TRAF6 complex in human CD34 ⁺ cells resulting in hematopoietic defects resembling MDS with del(5q). The overall objectives of this proposal are to (1) determine whether loss of TIFAB in human CD34 ⁺ cells contributes to MDS in mice; (2) to investigate whether deletions of TIFAB activate TRAF6 in MDS; and (3) to determine the consequences of TIFAB deletion on signal transduction in human CD34 ⁺ cells, and whether these could explain features of MDS. In preliminary data from the first 2 year of the proposal, we have evidence that TIFAB is a regulator of human hematopoietic cells. Our key observations show that knockdown of TIFAB in human CD34 ⁺ hematopoietic stem/progenitor cells results in increased survival and proliferation, TIFAB inhibits TRAF6 protein expression and activation, resulting in lower NF-kB activation, and TIFAB expression impacts leukemic cell survival, growth and progenitor function. Given that TIFAB is deleted in many MDS patients, these findings could have major implications in MDS subtypes with deletions of chr 5q. The observation that del(5q) results in inappropriate activation of TRAF6 provides a strong rationale to study the contribution of TIFAB to deregulation of the TRAF6 pathway in MDS.			

15. SUBJECT TERMS Myelodysplastic, BM Failure, TIFAB					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER <i>(include area code)</i>

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INTRODUCTION

Myelodysplastic syndromes (MDS) are clonal bone marrow failure (BMF) disorders defined by blood cytopenias due to ineffective hematopoiesis, genomic instability, and a predisposition to acute myeloid leukemia (AML). The most commonly recurring genomic alteration in MDS is deletion of chromosome 5q (del(5q)). MDS patients with an isolated del(5q) presenting with anemia, neutropenia, and elevated platelets associated with dysplastic megakaryocytes are considered to have 5q- syndrome. The majority of MDS patients with del(5q) do not exhibit these particular symptoms and, instead, are referred to as “del(5q) MDS”. We have recently identified miR-146a, which target the TRAF6 arm of the innate immune pathway, a gene within the deleted region in del(5q) MDS. We posit that multiple genes on chr 5q coordinate TRAF6 activation in del(5q) MDS. A search of annotated genes within or near the CDRs revealed a known inhibitor of TRAF6, TIFAB, on band q31.1. We hypothesize that deletion of TIFAB promotes activation of the TRAF6 complex in human CD34⁺ cells resulting in hematopoietic defects resembling MDS with del(5q). The overall objectives of this proposal are to (1) determine whether loss of TIFAB in human CD34⁺ cells contributes to MDS in mice; (2) to investigate whether deletions of TIFAB activate TRAF6 in MDS; and (3) to determine the consequences of TIFAB deletion on signal transduction in human CD34⁺ cells, and whether these could explain features of MDS. In preliminary data from the first 2 year of the proposal, we have evidence that TIFAB is a regulator of human hematopoietic cells. Our key observations show that knockdown of TIFAB in human CD34⁺ hematopoietic stem/progenitor cells results in increased survival and proliferation, TIFAB inhibits TRAF6 protein expression and activation, resulting in lower NF-κB activation, and TIFAB expression impacts leukemic cell survival, growth and progenitor function. Given that TIFAB is deleted in many MDS patients, these findings could have major implications in MDS subtypes with deletions of chr 5q. The observation that del(5q) results in inappropriate activation of TRAF6 provides a strong rationale to study the contribution of TIFAB to deregulation of the TRAF6 pathway in MDS.

BODY

Task 1. Plasmid constructs and validation (months 1-4):

1a (Complete). For knockdown of TIFAB in human CD34⁺ or MDS/AML cell lines, we used RNAi-mediated gene silencing. Lentiviral vectors encoding two independent shRNAs targeting human TIFAB were purchased from OpenBiosystems. The bicistronic lentiviral vector contains a microRNA-adapted shRNA and a CMV-driven turboGFP (Figure 1A)¹.

1c (Complete). For qRT-PCR and immunoblot analysis to determine TIFAB knockdown, two independent shRNAs targeting TIFAB (#88 and #89) and a scrambled control vector (shCTL) were transduced into human HL60 and THP1 leukemia cell lines. Two days post transduction, cells were sorted for GFP expression and expanded for an additional 2 days in culture. RNA and protein were collected and examined for TIFAB knockdown by qRT-PCR (Figure 1B) and immunoblotting (Figure 1C), respectively. In addition, we have also confirmed efficient knockdown in primary human cord blood CD34⁺ cells (Figure 2D).

1d. The original plan involved knocking down both miR-145 and miR-146a in primary hematopoietic cells using a miRNA decoy retroviral vector. Given that more recent findings indicate that miR-146a specifically targets the TRAF6 pathway and its deletion results in many

MDS-like features in mice^{2,3}, we have opted to clone only the miR-146a decoy. We have generated a decoy that knocks down the expression of miR-146a only. To confirm knockdown, we have transduced human CD34+ cells and performed qRT-PCR analysis for miR-146a knockdown. Unfortunately, the miRNA decoy is not efficient at knocking down miR-146a (Figure 1E) and does not affect survival (Figure 1F) in primary human CD34+ cells (after repeated attempts). Given the technical difficulties of simultaneously knocking down miR-146a and TIFAB, we have focused our attention on the posttranscriptional regulation of TRAF6 (See below).

Task 2. Expression analysis of TIFAB and TIFA in hematopoietic cells (months 1-6):

2a (Complete). Normal expression patterns of TIFA and TIFAB were investigated in human marrow subpopulations. This task is complete.

Task 3. Isolation and infection of CD34+ cells (months 4-18):

3a/b. Human umbilical cord CD34+ cells were obtained and cultured in vitro. CD34+ cells were transduced with shTIFAB-GFP, sorted, and evaluated in vitro. Transduction efficiency was 4% for shTIFAB. As indicated above, knockdown of TIFAB resulted in a reproducible downregulation of TIFAB mRNA by ~50%, which is consistent with the expression in del(5q) MDS patient cells (Figure 1D).

3c. As described above, the miR-146a decoy is not efficient at knocking down miR-146a in CD34+ cells despite reasonable transduction efficiency. Given that we are not able to progress with the miR-146a knockdown experiments, we have extended our analysis of TIFAB knockdown to a preleukemic CD34+ cells expressing AML1-ETO fusion (AE)⁴. Since AE CD34+ cells are not fully immortalized, we will determine whether knockdown of TIFAB in AE CD34+ cells will make them malignant. The same assays will be performed as proposed for normal CD34+ experiments (Task 4 and 5).

Task 4. Application of *in vitro* hematopoietic assays (months 8-12):

4a (Complete). Transduced shTIFAB (and control) CD34+ cells from Task 3 were used to determine the (i) proliferation, (ii) survival, and (iii) clonogenic potential in methylcellulose containing differentiation cytokines (IL3, IL6, SCF, and Epo).

- (i) For proliferation assays, transduced and sorted CD34+ cells were cultured in vitro and evaluated for trypan blue exclusion at the indicated times. Knockdown of TIFAB resulted in increased proliferation of CD34+ cells (Figure 2A).
- (ii) For survival assays, transduced and sorted CD34+ cells were cultured for 4 days and then evaluated for AnnexinV staining. As show in in Figure 2B knockdown of TIFAB resulted in a significant reduction of apoptotic (AnnexinV+) cells (Figure 2B).
- (iii) For clonogenic potential, transduced and sorted CD34+ cells were plated into methylcellulose and then scored for colony formation after 10 days. The number of colonies formed following knockdown of TIFAB was similar as compared to control-transduced cells (Figure 2C). When the proportion of colony types was examined, knockdown of TIFAB resulted in slight expansion of CFU-GM colonies at the expense of CFU-G colonies (Figure 2C); however,

these differences are not significant. Therefore, we conclude that knockdown of TIFAB does not alter the differentiation of CD34+ cells.

- (iv) For in vitro differentiation, transduced CD34+ were cultured for ~ 7 days in StemSpan media containing 10ng/mL IL3, IL6, TPO, FLT3, and SCF. Immature (CD34+) and mature myeloid (CD33+) cell surface expression was determined by FACS. As shown in Figure 2D, knockdown of TIFAB did not maintain cells in the immature CD34+ state. Conversely, CD34+ cells with knockdown of TIFAB exhibited an increase of mature CD33+CD34- cells.

These observations suggest that lower levels of TIFAB increase survival and proliferation of CD34+ cells but does not promote/maintain immature CD34+ cells.

Task 5. Application of bone marrow transplantation assays and analysis (months 8-36):

5a. We have transduced CD34+ cells with shCTL and shTIFAB and are ready for transplantation into NSGS mice (NOD/SCID mouse with IL2R γ knockout and transgenic expression of SCF, GM-CSF, and IL-3)⁵. The NSGS colony is maintained by our laboratory and ready for xenotransplantation. For the first experiment, we are prepared to engraft ~10 mice per group (shCTL and shTIFAB). Mice will be analyzed according to our original plan (Task 5b-5d). In addition, we will xenograft AE-CD34+ cells transduced with shCTL or shTIFAB. As for xenotransplantation of normal CD34+ cells, mice receiving AE-CD34+ will be monitored for MDS/AML-like features and hematopoietic defects.

Task 6. Identification of changes in TRAF6 activation and NF- κ B signaling by TIFAB (months 10-14):

It was previously reported that TIFAB functions by suppressing TRAF6-mediated NF- κ B activation^{6,7}. However, the mechanism by which TIFAB may inhibit TRAF6 and/or NF- κ B is not known. Based on our data from Year 1, we find that TIFAB reduces the TRAF6 protein and function. One of the key findings was that TIFAB suppressed LPS-induced NF- κ B activation but not TNF-induced NF- κ B activation. This observation is consistent with the finding that TIFAB inhibits TRAF6 function and stability. Based on this interesting finding, the experiments in Year 2 were focused on determining the effects of TIFAB on TRAF6-induced NF- κ B activation.

6a. We have measured IL-6 and BFL1-A1 (two NF- κ B target genes) in CD34+ cells transduced with shTIFAB (Figure 3A). We observed an ~1.5 increase in IL6 expression but no significant changes in BFL1A1 in cells with knockdown of TIFAB. This initial observation suggests that TIFAB does not regulate all functions of NF- κ B signaling. We are currently evaluating other NF- κ B target genes, and determining the activation and expression level of TRAF6 in CD34+ cells with knockdown of TIFAB.

6b. We have devoted a significant effort to dissect the role of TIFAB on NF- κ B activation. Our initial efforts have been performed in HEK293 cells using NF- κ B luciferase reporter assays:

- i. To investigate the dosage effects of TIFAB on TNF or LPS-induced NF- κ B activation, we transfected increasing amounts of pcDNA-TIFAB into HEK293 cells and then stimulated with TNF (1 ng/ml) or LPS (1 ug/ml). As shown in Figure 3C, TIFAB (even at the lowest dose) efficiently inhibited LPS-induced kB-site luciferase activity, but only inhibited TNF-induced kB-luciferase at the highest dose (Figure 3B). Conversely, increasing the amount of LPS did not overcome the inhibitory effects of TIFAB on NF- κ B activation (Figure 3D).

- ii. To investigate the dosage effects of TRAF6 on TIFAB repression of NF- κ B, we transfected increasing amounts of pcDNA-TRAF6 and pcDNA-TIFAB into HEK293 cells. At all doses examined, TIFAB expression suppressed TRAF6-mediated κ B-site activation (Figure 3E).
- iii. To further understand the specificity of TIFAB-mediated inhibition of NF- κ B, we cotransfected various NF- κ B activators and then assessed the effects of TIFAB (Figure 4A). As shown before, TIFAB effectively inhibits TRAF6-mediated activation of κ B site-luciferase (Figure 4B). Since TRAF6 shares functional and sequence homology with TRAF2, we examined the effects of TIFAB on TRAF2-mediated activation of NF- κ B (Figure 4C). Interestingly, TIFAB was not able to inhibit TRAF2-mediated κ B site-luciferase activity. Since we hypothesize that TIFAB directly inhibits TRAF6, we wanted to determine whether transfecting in IKK (NF- κ B kinase) or p65 (NF- κ B transcription factor), which are both downstream of TRAF6, could still maintain κ B site-luciferase activity in the presence of TIFAB (Figure 4A). As expected, IKK or p65 induced κ B site-luciferase activity even in the presence of TIFAB (Figure 4D, 4E). These findings reinforce the hypothesis that TIFAB directly and specifically inhibits TRAF6-mediated NF- κ B activation.
- iv. To corroborate the NF- κ B reporter assays, we investigated the consequences of TIFAB expression on TRAF6 by performing immunoblots (Figure 4F). As shown, TIFAB expression results in less TRAF6 protein.
- v. TIFAB is 161 amino acids (aa) and contains a fork-head associated domain (FHA) spanning ~ aa 36-91 (Figure 5A). To identify the critical region for TIFAB-mediated inhibition of TRAF6, we created TIFAB deletion mutants (Figure 5A). Each mutant was fused to the FLAG epitope to conveniently detect expression by immunoblotting. Following transfection into HEK293 cells, the protein expression of the TIFAB deletion mutants was confirmed by FLAG immunoblots (Figure 5B). The consequences of TIFAB domain deletions on basal NF- κ B activation were first evaluated without TRAF6 expression. As shown in Figure 5C, TIFAB D1-34, D92-161, and D35-161 did not inhibit basal κ B-site luciferase activity; in contrast, the level of NF- κ B appeared to increase when these mutants were expressed (Figure 5C). Mutant D1-91 inhibited basal NF- κ B activation (Figure 5C).
- vi. Next the TIFAB mutants were evaluated for their ability to repress TRAF6-mediated κ B site-luciferase activity. As before, full-length TIFAB repressed TRAF6-mediated κ B site activity (Figure 5D). Similar to the trend observed for basal NF- κ B repression, TIFAB D1-34, D92-161, D36-91, and D35-161 did not efficiently repress TRAF6-mediated κ B site activity (Figure 5D). However, D1-91 significantly repressed TRAF6-mediated κ B site activity (Figure 5D). To extend these observations, we evaluated TRAF6 protein expression following transfection of the TIFAB mutants. Consistent with the κ B-luciferase assays, D35-91 and D1-91 reduced endogenous TRAF6 expression (Figure 5E). These findings suggest that the inhibitory domain of TIFAB resides within aa 92-161. To further define the minimal sequence necessary to inhibit TRAF6, we have created additional TIFAB mutants: D1-125, D1-91+D126-161, D126-161, and D91-126 (Figure 5A). These mutants are currently being evaluated.

Task 7. Gene expression analysis (months 16-24).

This task as not been initiated.

Task 8. Validation of targets (months 24-36)

This task as not been initiated.

Extra Tasks: Investigating the function of TIFAB by identifying interacting proteins.

The function of TIFAB is not well defined. To further identify the molecular role of TIFAB in MDS/AML cells, we performed a proteomics experiment to identify proteins that bind to TIFAB. For these experiments, we created a FLAG-tagged TIFAB cDNA in a retroviral vector. HL60 cells were transduced with vector or FLAG-TIFAB. HL60 cells expressing vector or TIFAB were immunoprecipitated with FLAG-containing beads. FLAG-TIFAB complexes were eluted off the beads and run on a polyacrylamide gel for silver stain analysis. Bands that were unique to the lane containing FLAG-TIFAB (and the control bands) were cut from the gel and evaluated by mass spectrometry (box 5 versus 4, and 7 versus 6) (Figure 6A). We also cut the band coinciding with the molecular weight of TIFAB (box 8). Based on the mass spectrometry analysis, box 8 contains TIFAB peptides (Figure 6B). In addition, we identified unique bands in Box 5 corresponding to Myeloperoxidase (PERM/MPO) (Figure 6B). Based on this initial analysis, we have identified at least one unique TIFAB-interacting protein. Future experiments will validate the interaction between TIFAB and MPO and the consequences of this interaction on TRAF6 function. MPO, a lysosomal protein, functions in the host defense system by catalyzing the production of hypohalous acids. Since our recent publication revealed that TRAF6 protein is regulated by lysosomes, we will test the hypothesis that TIFAB-mediated inhibition of TRAF6 occurs through a lysosomal pathway.

KEY RESEARCH ACCOMPLISHMENTS

- Identified an shRNA lentiviral vector containing a GFP reporter that knocks down the expression of human TIFAB by approximately 50% in human CD34+ cells.
- Successfully transduced normal CD34+ cells and human leukemic cell lines to evaluate TIFAB knockdown with the shRNA.
- Determined that knockdown of TIFAB in human CD34+ cells resulted in increased CD34+ survival and increased proliferation, without affecting differentiation potential.
- Discovered that TIFAB suppresses active TRAF6 and reduces TRAF6 protein expression in human cell lines.
- Determined that TIFAB suppress NF- κ B activation following LPS/TRAF6-mediated stimulation, but not via TRAF2/TNFR.
- Narrowed the region of TIFAB that possess the inhibitory function (between aa 91-161).
- Identified a novel TIFAB interacting protein by mass spectrometry.

REPORTABLE OUTCOMES

Accepted Research Article:

Guo F, S Zhang, M Grogg, JA Cancelas, ME Varney, **DT Starczynowski**, W Du, J Yang, W Liu, G Thomas, S Kozma, Q Pang, Yi Zheng. Mouse gene targeting reveals an essential role of mTOR in hematopoietic stem cell engraftment and hematopoiesis. (2013). *Haematologica*, in press. May 28, 2013.

Rhyasen G, L Bolanos, J Fang, C Rasch, A Jerez, M Varney, M Wunderlich, C Rigolino, L Mathews, M Ferrer, N Southall, R Guha, J Keller, C Thomas, L Beverly, A Cortelezzi, EN Oliva, M Cuzzola, J Maciejewski, JC Mulloy, **DT Starczynowski**. Targeting IRAK1 as a novel therapeutic approach for Myelodysplastic Syndrome. (2013). *Cancer Cell*, in press. June 8, 2013.

Abstracts at Conferences:

Melinda E. Varney, Andres Jerez, Jing Fang, David Miller, Lyndsey Bolanos, Aly Karsan, Jaroslaw P. Maciejewski, Daniel T. Starczynowski. American Society of Hematology, Atlanta, GA, December, 2012.

Melinda E. Varney, Andres Jerez, Jing Fang, David Miller, Lyndsey Bolanos, Aly Karsan, Jaroslaw P. Maciejewski, Daniel T. Starczynowski. Ohio State University Cancer Center Conference, April, 2013.

Awards:

ASH 2012 Abstract Achievement Award (for Melinda Varney)

CONCLUSIONS

The first year of the project has yielded many interesting results, most of which support our original hypothesis, and allowed us to continue with the majority of goals in the Statement of Work. Overall, the experiments suggest that TIFAB, a novel and uncharacterized protein, exhibits tumor suppressor-like functions in human hematopoietic cells. Our key observations show that (1) TIFAB is primarily expressed in hematopoietic progenitor cells (rather than in primitive hematopoietic stem cells or mature blood cells); (2) knockdown of TIFAB in human CD34+ hematopoietic stem/progenitor cells results in increased survival and altered hematopoietic progenitor function; (3) TIFAB inhibits TRAF6 protein expression and activation, resulting in lower NF- κ B activation; and (4) TIFAB expression impacts leukemic cell survival, growth and progenitor function. Given that TIFAB is deleted in many MDS patients, these findings could have major implications in MDS and AML subtypes with deletions of chr 5q.

As indicated above, majority of the goals in the Statement of Work have been accomplished for the first 2 years of the proposal. In addition, our ongoing experiments support our hypothesis, permitting us to continue with our original outline of experiments. However, there have been a few minor alterations to the Statement of Work:

- We have limited our analysis of TIFA as it appears that TIFAB has a major effect on TRAF6 independent of TIFA levels. In addition, TIFA expression did not differ in

hematopoietic subpopulations. As such, we propose to delay examining TIFA's potential role in linking TIFAB and TRAF6.

- Based on our preliminary findings, knockdown of miR-146a does not appear to be feasible in primary human CD34+ cells. As such we have opted to temporarily not focus on miR-146a until we find an alternative strategy. In place of these original experiments, we propose to focus on the direct inhibitory function of TIFAB on TRAF6 by performing detailed structure functional analysis and identification of novel TIFAB interacting proteins.
- Since we have optimized consistent knockdown of TIFAB in primary CD34+ cells, we will also investigate the consequences of knocking down TIFAB in preleukemic CD34+ that contain an AML1-ETO fusion gene. As with primary CD34+ cells, we will determine the effects of TIFAB knockdown on proliferation, progenitor function, survival, and xenotransplantation in NSGS mice.

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3. Boldin, M.P. *et al.* miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med* **208**, 1189-201 (2011).
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APPENDICES

- Figures 1-5
- Curriculum Vitae

Figure 1.

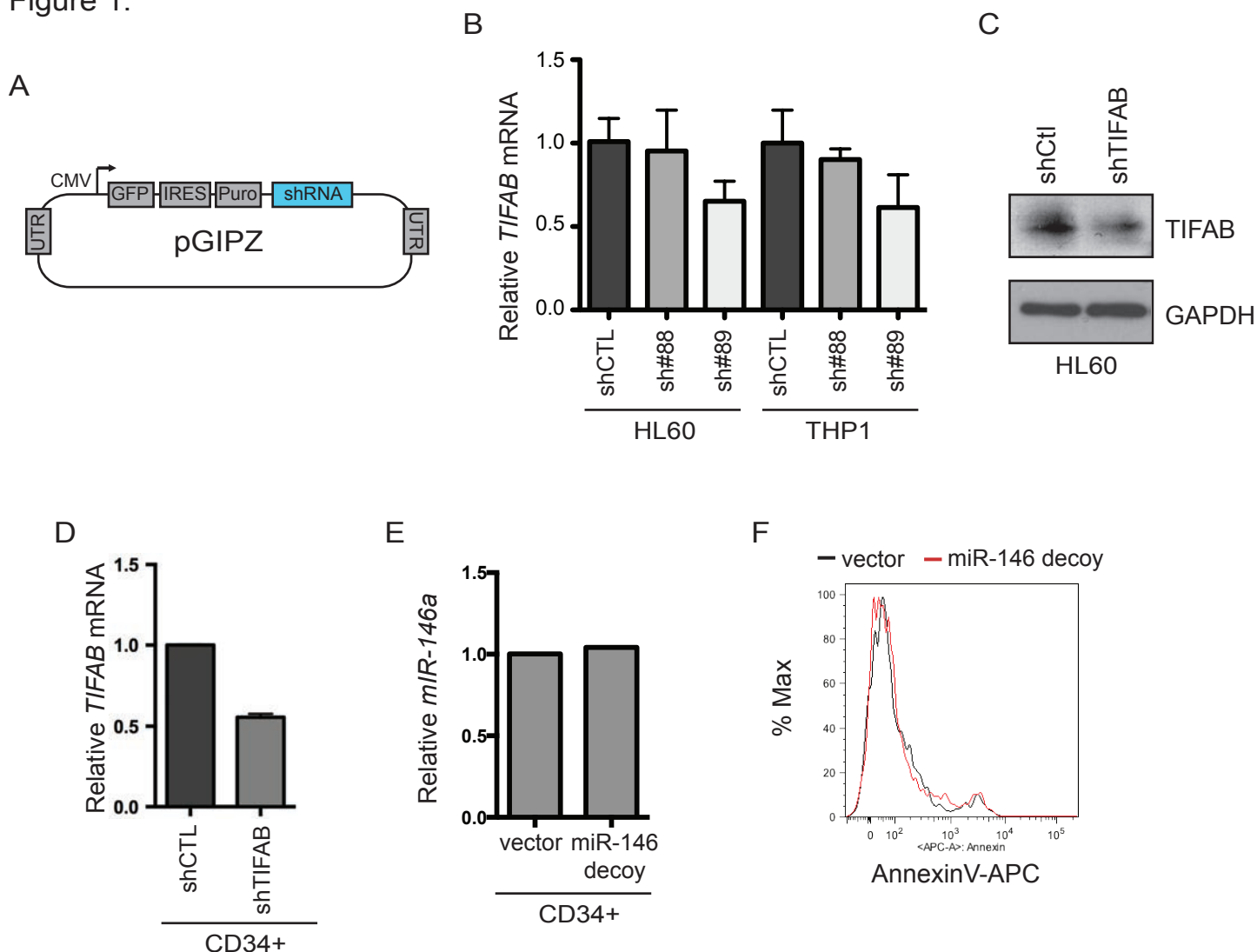


Figure 1: Task 1

A. Schematic representation of the pGIPZ lentiviral vector for knockdown of TIFAB is shown. On The bicistronic miRNA-based shRNA is driven by a CMV promoter and also expresses GFP for tracking expression in transduced cells. For simplicity, cPPT, WRE, Amp, pUC, and Ori are not shown.

B. qRT-PCR was performed on HL60 and THP1 cells transduced with the indicated shRNA-containing lentiviral vectors.

C. THP1 cells were transduced with control shRNA or shTIFAB and analyzed for TIFAB expression by immunoblotting with the indicated antibodies.

D. qRT-PCR was performed on primary CD34+ cord blood cells transduced with the indicated shRNA-containing lentiviral vectors.

E. pGK-miR-146a-GFP decoy was transduced into CD34+ cells. RNA was collected and analyzed by qRT-PCR for miR-146a expression.

F. CD34+ cells transduced with miR-146a decoy were evaluated for AnnexinV staining.

Figure 2.

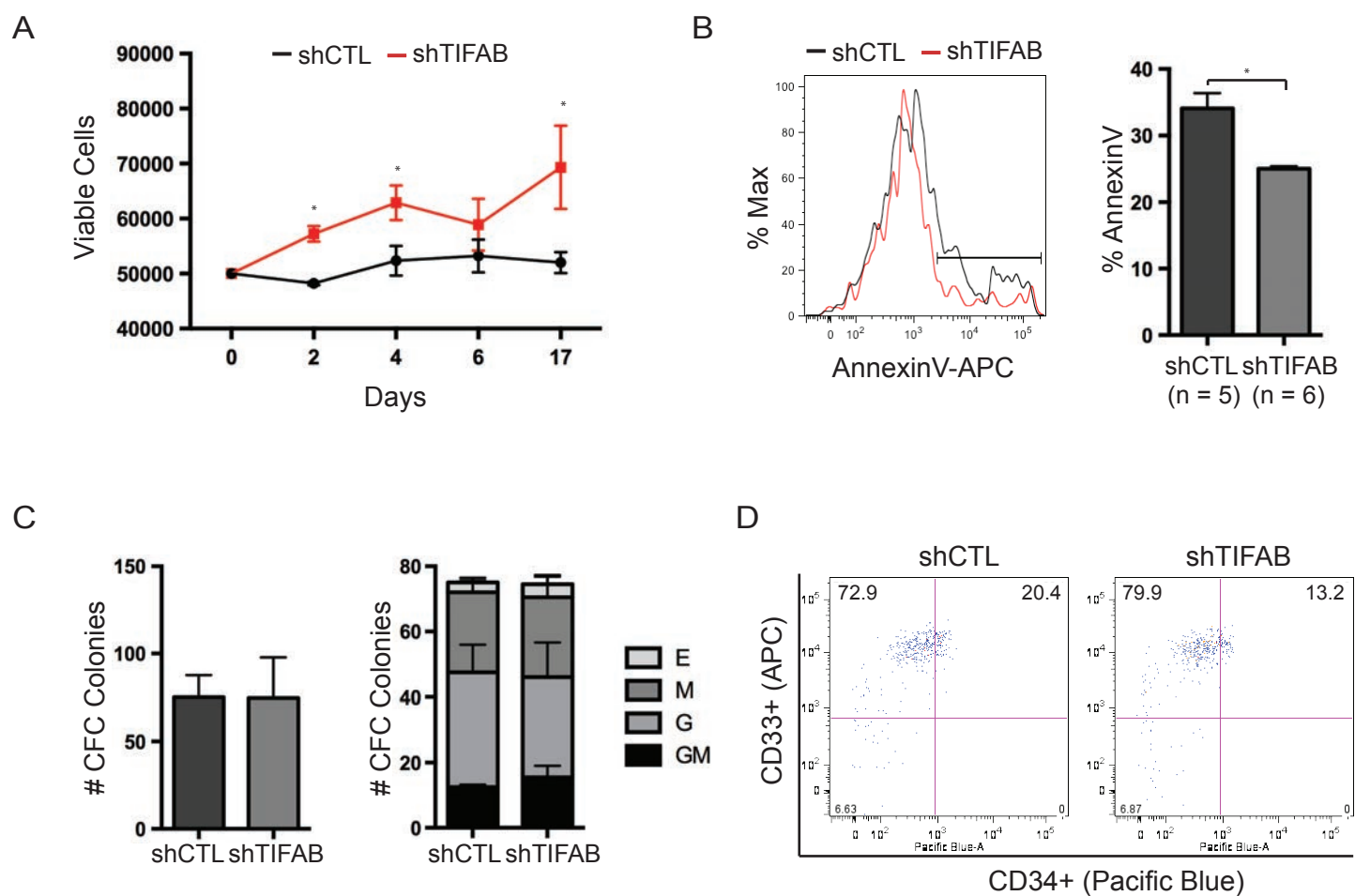


Figure 2: Task 4

A. 1×10^6 human CD34⁺ cells were transduced with control shRNA or shTIFAB (clone #89) and then sorted for GFP⁺ cells. 5×10^4 transduced cells were cultured in vitro and counted at the indicated days.

B Transduced CD34⁺ cells were evaluated for AnnexinV staining. The histogram is the summary of 2 independent transductions.

C. 5×10^4 transduced CD34⁺ were plated in methylcellulose and analyzed for colony formation. Colonies were scored after 14 days. E, erythroid; M, monocyte; G, granulocyte.

D. Transduced CD34⁺ cells were evaluated for CD34 and CD33 staining after 7 days in culture.

Figure 3.

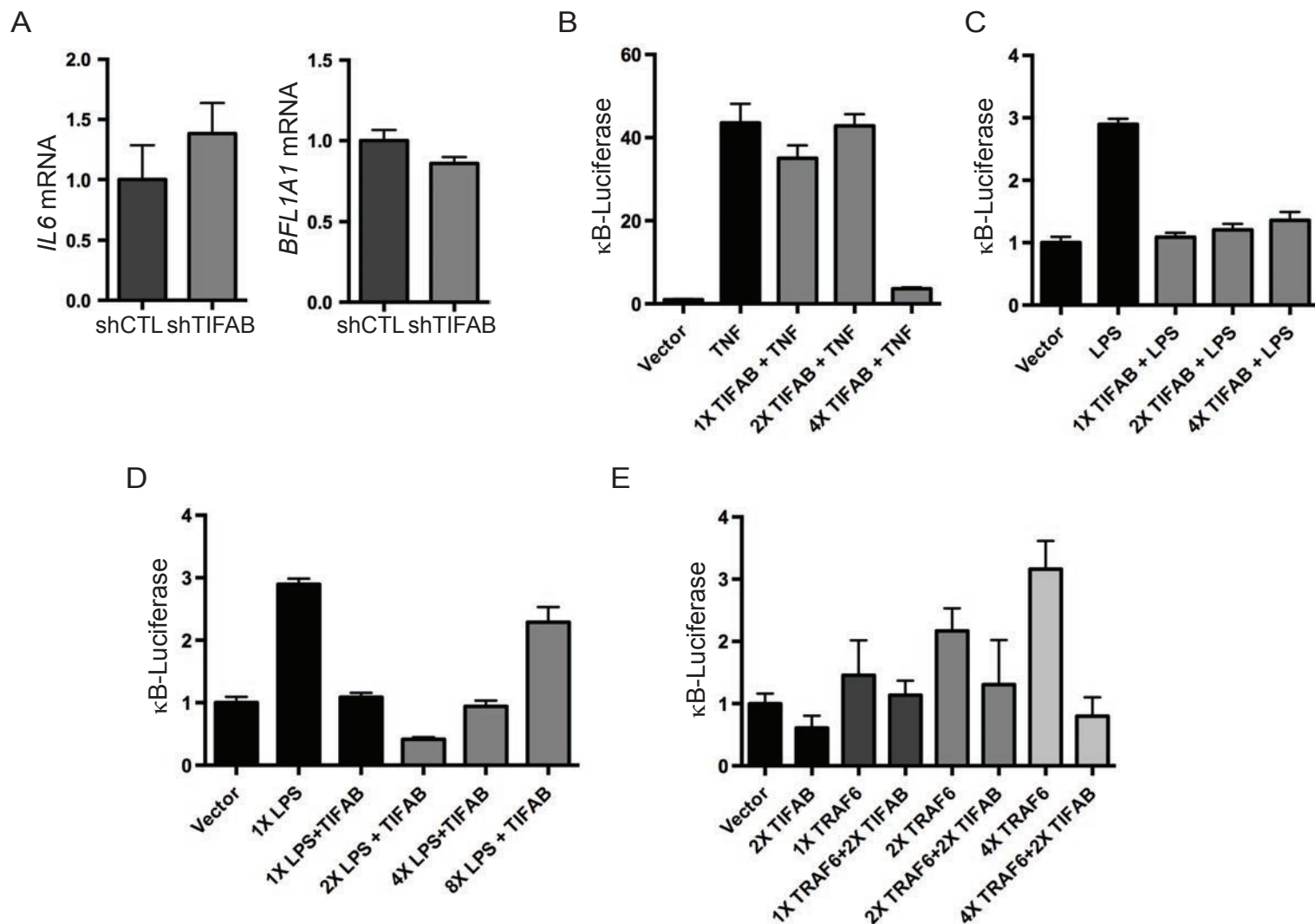


Figure 3: Task 5

A. qRT-PCR was performed for IL6 and BFL1A1 expression in human CD34+ transduced with the indicated vectors.

B. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng) or pcDNA3.1-FLAG-TIFAB (250, 500, or 1000 ng) and κB-luciferase. Following transfection, cells were simulated with TNFα (1 ng/ml) for 6 hours. Values represent relative luciferase.

C. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng) or pcDNA3.1-FLAG-TIFAB (250, 500, or 1000 ng) and κB-luciferase. Following transfection, cells were simulated with LPS (1 ug/ml) for 6 hours. Values represent relative luciferase.

D. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng) or pcDNA3.1-FLAG-TIFAB (250 ng) and κB-luciferase. Following transfection, cells were simulated with LPS (1, 2, 4, or 8 ug/ml) for 6 hours. Values represent relative luciferase.

E. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-TIFAB (250, 500, or 1000 ng), and/or pcDNA3.1-FLAG-TRAF6 (250, 500, or 1000 ng) and κB-luciferase. Values represent relative luciferase.

Figure 4.

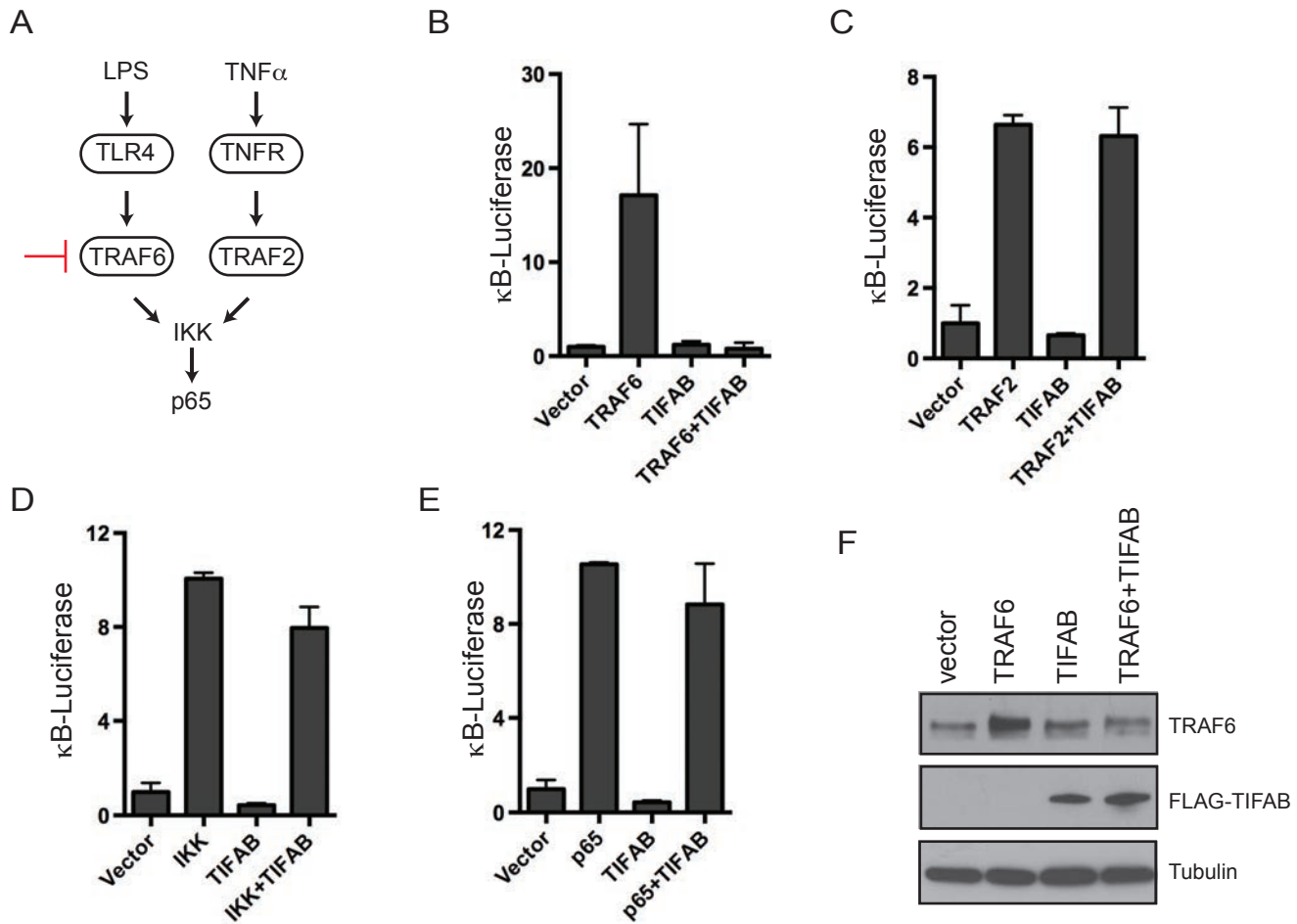


Figure 4: Task 6b

A. Model distinguishing LPS/TLR4 and TNF α /TNFR activation of NF- κ B. We propose that TIFAB selectively inhibits TRAF6.

B. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-TRAF6 (250 ng) and/or pcDNA3.1-FLAG-TIFAB (250 ng) and κ B-luciferase. Following transfection, cells were simulated with TNF α (1 ng/ml) for 6 hours. Values represent relative luciferase.

C. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-TRAF2 (250 ng) and/or pcDNA3.1-FLAG-TIFAB (250 ng) and κ B-luciferase. Following transfection, cells were simulated with TNF α (1 ng/ml) for 6 hours. Values represent relative luciferase.

D. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-IKK β (250 ng) and/or pcDNA3.1-FLAG-TIFAB (250 ng) and κ B-luciferase. Following transfection, cells were simulated with TNF α (1 ng/ml) for 6 hours. Values represent relative luciferase.

E. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-p65/RelA (250 ng) and/or pcDNA3.1-FLAG-TIFAB (250 ng) and κ B-luciferase. Following transfection, cells were simulated with TNF α (1 ng/ml) for 6 hours. Values represent relative luciferase.

F. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-TRAF6 (250 ng) and/or pcDNA3.1-FLAG-TIFAB (250 ng). Following transfection, lysates were collected and evaluated by immunoblotting.

Figure 5.

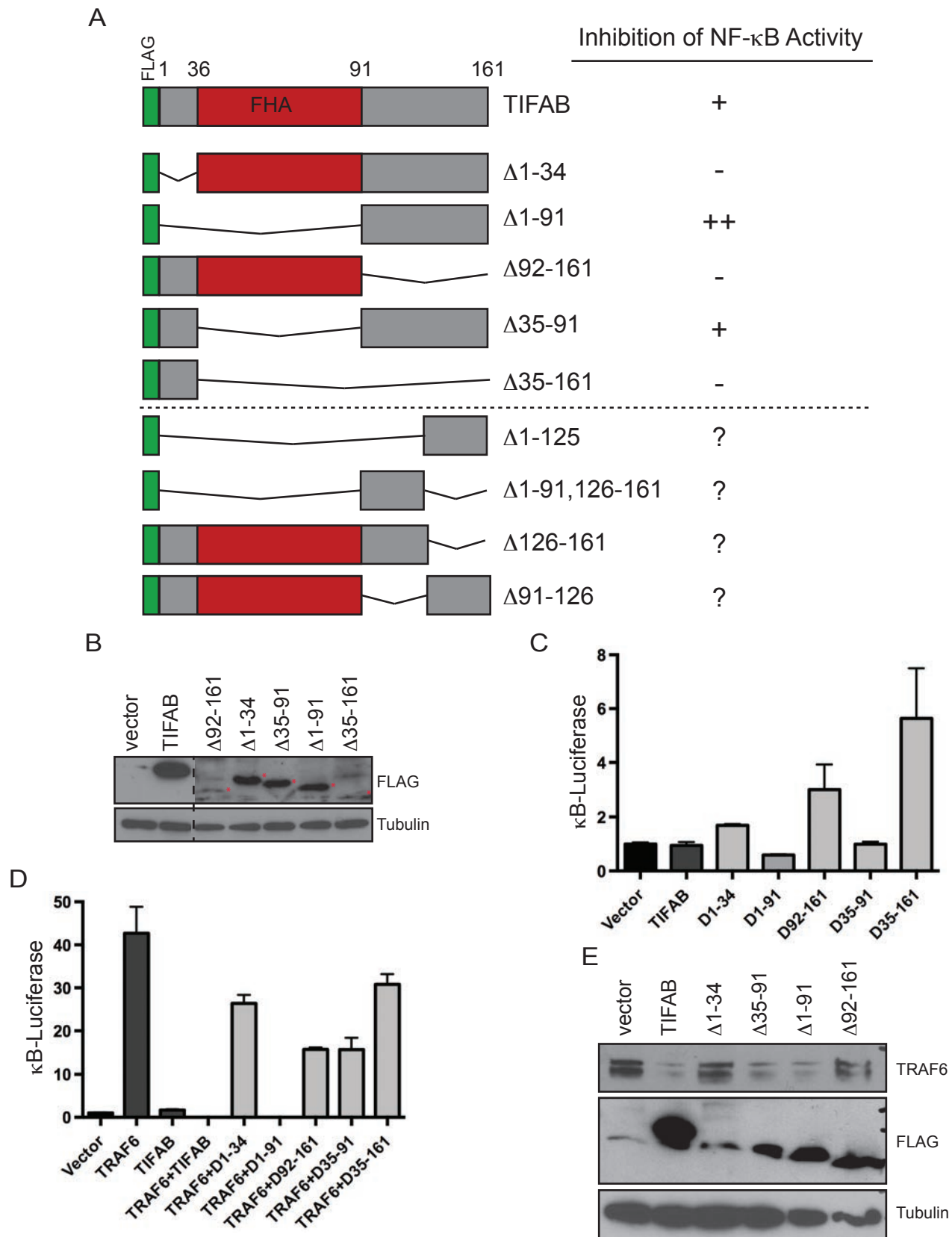


Figure 5: Task 6c

A. Structure of wild-type and TIFAB deletion mutants.

B. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-TIFAB (250 ng) or the indicated TIFAB deletion mutants. Following transfection, protein lysates were collected and evaluated by immunoblotting with an anti-FLAG antibody.

C. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-TIFAB (250 ng), or the TIFAB deletion mutants, and κ B-luciferase. Values represent relative luciferase.

D. HEK293 cells were transfected with pcDNA3.1 (vector, 250 ng), pcDNA3.1-FLAG-TRAF6 (250 ng) and pcDNA3.1-FLAG-TIFAB (250 ng) or TIFAB deletion mutants, and κ B-luciferase. Values represent relative luciferase.

E. HEK293 cells were transfected with the indicated vectors and then evaluated by immunoblotting.

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Personal Statement

Research in my laboratory focuses on the molecular, cellular, and genetic basis of hematologic malignancies, with a specific focus on Myelodysplastic Syndromes (MDS) and Acute Myeloid Leukemia (AML). We have uncovered aberrant activation of the innate immune pathway in MDS, and now find that this activation perturbs normal hematopoietic stem/progenitor (HSPC) function. Therefore, one of the main objectives of my research program is to evaluate the role of the innate immune pathway in normal HSPC and in MDS. Broader initiatives involve characterizing novel candidate genes (including miRNAs) relevant to MDS, dissecting molecular and cellular alterations in MDS (and related hematologic malignancies), and attempting to identify novel therapeutics. In addition, I have extensive experience and ongoing interests in NF- κ B signaling and function in immune and stem cells.

Education and Training

2005-2010	Postdoctorate BC Cancer Research Centre/University of British Columbia, Vancouver, Canada
2000-2005	Ph.D., Molecular Biology, Cell Biology and Biochemistry Boston University, Boston, MA, USA.
1996-2000	B.Sc., Honors Biology Concentrations in Chemistry and Biotechnology Fairleigh Dickinson University, Teaneck, NJ, USA.

Research and Professional Experience

2010- present	Assistant Professor, Division of Experimental Hematology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH
2010-present	Affiliate Assistant Professor, Department of Cancer and Cell Biology, University of Cincinnati, Cincinnati, OH
2010- 2011	Lecturer, Ulm University, Germany
2005-2010	Postdoctoral Fellow Research Focus: <i>Identification and functional analysis of genetic and molecular determinants of hematological malignancies: deregulation of miR-146/TRAF6 signaling in Myelodysplastic syndromes</i> Advisor: Dr. Aly Karsan, BC Cancer Research Centre/University of British Columbia
2001-2005	Graduate Research Assistant

Dissertation: *A mutational and functional analysis of C-terminal sequences of transcription factor REL: their role in cellular transformation and transcriptional activation*
Advisor: Dr. Thomas D. Gilmore, Boston University

- 2000 Summer Research Assistant
Research Focus: *Functional analysis of androgen receptor in prostate cancer*
Advisor: Dr. Marianne Sadar, BC Cancer Research Centre
- 1999-2000 Honors Research
Dissertation: *Inhibitory effects of somatostatin on the viability of a cell line*
Advisor: Dr. Anjali Saxena, Fairleigh Dickinson University
- 1997-1999 Research Assistant
Research Focus: *Development of enzyme-friendly biosensors for lactate detection*
Advisor: Dr. Mihaela Leonida, Fairleigh Dickinson University

Teaching Experience

- 2013 Lectures
“Critical data presentation III”, Department of Cancer and Cell Biology, University of Cincinnati.
Participated in primary research evaluation and interpretation with graduate students.
- 2012 Course
“Introduction to Grant Writing”, Department of Cancer and Cell Biology, University of Cincinnati.
Developed and co-taught a course on basic grant writing fundamentals, experience putting them in practice, and responding to critiques. The course is offered to graduate students and implements traditional didactic and web-based components.
- 2012 Lectures
“Critical data presentation III”, Department of Cancer and Cell Biology, University of Cincinnati.
Participated in primary research evaluation and interpretation with graduate students.
- 2010 Lectures
“Innate Immunity and Cancer”, Master Online Program in Advanced Oncology. Ulm University.
Developed a video-based lecture on innate immunity and its role in human cancer for postgraduates of medicine.
- 2010 Individual Lecture
“MicroRNAs in cancer”, Medical Oncology Residency Training Program. BC Cancer Agency.
Organized and conducted a lecture for 1st and 2nd year residents of medical training on microRNAs and their role in clinical oncology.
- 2009 Individual Lecture
“MicroRNAs in hematological malignancies”, Hematology Fellows Series. Department of Medicine, University of British Columbia.
Organized and conducted a lecture for hematology and pathology fellows on the emerging role of microRNAs in hematological malignancies.
- 2009 Workshop
“MicroRNAs: small RNAs with big impact”. Terry Fox Laboratory, BC Cancer Research Centre.
Organized and co-lead a workshop on microRNAs, their biogenesis, diversity, and mechanism as related to gene regulation and to human disease.

- 2005 Teaching Assistant
 “Molecular Biology Laboratory”, Boston University.
Prepared and delivered weekly labs for advanced (500-level) molecular biology students.
- 2000-2001 Teaching Fellow
 “Life Sciences Chemistry I and II”, Boston University
Organized and delivered weekly labs for introductory (100-level) undergraduate science students to supplement course material. Prepared and evaluated exams and final grades. Held weekly office hours.
- 1998 Teaching Assistant
 “Chemistry for Health Science”, Fairleigh Dickinson University.
Organized and delivered weekly labs for introductory (100-level) undergraduate science students.

Awards and Honors

- 2013-2016 Gabrielle’s Angel Foundation Medical Research Award
- 2011-14 American Society of Hematology Scholar Award, Basic Research Junior Faculty
- 2010 National Institute of Allergy and Infectious Disease, Keystone Symposium Scholarship
- 2008 Travel Award, American Society of Hematology
- 2008 Eugene Cronkite Award: New Investigator Award (1st place).
 International Society of Experimental Hematology
- 2008 Travel Grant, International Society of Experimental Hematology Scientific Meeting
- 2007-2010 Postdoctoral Fellowship, Canadian Institute of Health Research
- 2006 Frank A. Belamarich Award; Outstanding Scholarship and Performance in Graduate Studies,
 Biology Department, Boston University
- 2006-2009 Postdoctoral Fellowship, Michael Smith Foundation for Health Research
- 2001-2005 Graduate Research Fellowship, Boston University
- 2002-2004 Postgraduate Research Scholarship, Natural Sciences and Engineering Research Council of Canada
- 2002 Travel Scholarship, NF- κ B: Bench to Bedside - Keystone Symposium
- 2000-01, 2005 Teaching Fellowships, Boston University
- 2000 J.M. Warren Summer Research Scholarship, British Columbia Cancer Agency
- 2000 ECAC Award: Graduating athlete with highest cumulative grade point average, Fairleigh Dickinson University, NJ
- 2000 Summa Cum Laude, Fairleigh Dickinson University

2000	Phi Omega Epsilon, Honor Society
1999	Phi Zeta Kappa, Honor Society
1999	Charter Day Scholarship, Fairleigh Dickinson University
1998	University-College Dean's Award, Fairleigh Dickinson University
1996-2000	University Honors List, Fairleigh Dickinson University
1996-2000	Tennis Scholarship, Fairleigh Dickinson University

Memberships and Committees

2013	Scholar Awards Study Section Reviewer, American Society of Hematology
2013	Grant Reviewer, Swiss Cancer League
2012	Scholar Awards Study Section Reviewer, American Society of Hematology
2012-present	Early Career Reviewer (ECR) at the Center for Scientific Review, National Institute of Health
2011 (Sept)	NIH State of the Science Symposium: Myelodysplastic Syndrome Working Group, Bethesda MD (Invited member)
2011-present	Graduate student recruitment committee, Cancer and Cell Biology Graduate Program, University of Cincinnati
2011-present	Immunobiology Graduate Program, Cincinnati Children's Hospital (Training Faculty)
2010-present	Cancer and Cell Biology Graduate Program, University of Cincinnati (Training Faculty)
2008	Campus Provost Search Committee, Fairleigh Dickinson University-Vancouver
2008-present	International Society of Experimental Hematology (Member)
2005-present	American Society of Hematology (Associate Member)
2006-2011	Board of Directors, Fairleigh Dickinson University-Vancouver (Member)
1999-2000	<i>TriBeta</i> Biological Society, Fairleigh Dickinson University Chapter (Vice President)
1997-2000	University Honors Program, Fairleigh Dickinson University

University Service

2013	Midwest Blood Club Annual Meeting (Co-Organizer)
2013	ASBMB Career Symposium (Planning Committee Member)
2013	Office of Postdoctoral Affairs, CCHMC (Assistant Director)

2012	Qualifying Exam Committee, Graduate Program in Cancer and Cell Biology, University of Cincinnati (Standing Member)
2011 (Sept)	CCB Graduate Program Student Symposium, University of Cincinnati (Faculty co-organizer)
2011 (Feb)	Research Ethics, University of Cincinnati (Discussion leader)
2011-12	Cancer and Blood Diseases Institute Seminar Series (Co-coordinator)
2011	Scholarship Oversight Committee, Hematology/Oncology Clinical Fellowship (Committee Member)
2011	Immunohematology Club Seminar Series (Speaker)

Patents

Daniel Starczynowski and Garrett Rhyasen, “Combination therapy for MDS”, U.S. provisional patent, June 2013

Aly Karsan and Daniel Starczynowski, “TRAF6 as a therapeutic target and predictive biomarker for lung and colorectal cancer,” U.S. provisional patent, September 2010

Aly Karsan and Daniel Starczynowski, “Novel methods to predict therapeutic response to Lenalidomide and related drugs”, U.S. provisional patent, January 2008

Peer-Reviewed Publications

1. Gilmore TD, M-E Gapuzan, D Kalaitzidis, and **D Starczynowski**. (2002) Rel/NF-kB/IkB signal transduction in the generation and treatment of human cancer. Cancer Letters, 181: 1-9. PMID:12430173 (Review)
*Top 25 Hottest Article” on ScienceDirect within Cancer Letters (April-June 2005)
2. Leonida MD, **DT Starczynowski**, R Waldman, and B Aurian-Blajeni. (2003) Polymeric FAD used as enzyme-friendly mediator in lactate detection. Analytical and Bioanalytical Chemistry, 376: 832-837. PMID: 12811450
3. **Starczynowski DT**, JG Reynolds, and TD Gilmore. (2003). Deletion of either C-terminal transactivation subdomain enhances the *in vitro* transforming activity of human transcription factor REL. Oncogene, 22: 6929-6936. PMID: 14534540
4. Gilmore TD, D Kalaitzidis, M-C Liang, and **DT Starczynowski**. (2004). The c-Rel transcription factor and B-cell proliferation: a deal with the devil. Oncogene, 23: 2275-2286. PMID: 14755244 (Review)
5. Gilmore TD, D Kalaitzidis, and **DT Starczynowski**. (2004). *RE*levant gene amplification in B-cell lymphomas. Blood. 103: 3243. PMID: 15070712 (Letter)
6. Kalaitzidis D, J Ok, L Sulak, **DT Starczynowski**, and TD Gilmore. (2004). Characterization of a human REL-estrogen receptor fusion protein with a reverse conditional transforming activity in chicken spleen cells. Oncogene, 23: 7580-7587. PMID:15326488
7. **Starczynowski DT**, JG Reynolds, and TD Gilmore. (2005). Mutations of tumor necrosis factor α -responsive serine residues 460 and 471 within the C-terminal transactivation domain of human transcription factor REL can enhance its *in vitro* transforming ability. Oncogene, 24: 7355-7368. PMID: 16027730

8. **Starczynowski DT**, H Trautmann, C Pott, L Harder, N Arnold, R Siebert, and TD Gilmore. (2007). Mutation of an IKK phosphorylation site within the transactivation domain of REL in two patients with B-cell lymphoma enhances REL's *in vitro* transforming activity. Oncogene, 26: 2685-2694. PMID: 17072339
*Oncogene's Featured Article (April 2007)
9. **Starczynowski DT**, S Vercauteren, S Sung, A Brooks-Wilson, J Spinelli, C Eaves, A Eaves, D Horsman, W Lam, and A Karsan. (2008). High-resolution array comparative genomic hybridization of CD34⁺ cells from patients with low-risk myelodysplastic syndromes predicts overall and leukemia-free survival. Blood, 112(8): 3412-3424. PMID: 18663149
10. **Starczynowski DT**, F Kuchenbauer, B Argiropoulos, S Sung, R Morin, A Muranyi, D Hogue, R Wells, M Marra, WL Lam, K Humphries, and A Karsan. (2010). Identification of miR-145 and miR-146a as microRNAs involved in the pathogenesis of 5q- syndrome. Nature Medicine, 16(1): 49-58. PMID: 19898489
*Comment in Nature Medicine. 2010. Myelodysplasia: Battle in the Bone Marrow. 16(1): 30-32.
*Featured in The Hematologist. 2010. Clarifying the genetic underpinnings of the 5q- syndrome. July/Aug:7(4).
11. **Starczynowski DT** and A Karsan. (2010). Deregulation of innate immune signaling in myelodysplastic syndromes is associated with deletion of chromosome arm 5q. Cell Cycle, 11; 9(5). PMID: 20160505 (Review)
12. **Starczynowski DT** and A Karsan. (2010). Innate immune signaling in Myelodysplastic Syndromes. Hematology/Oncology Clinics of North America, 24(2):343-359. PMID: 20359630 (Review)
13. Vercauteren SM, S Sung, **DT Starczynowski**, WL Lam, H Bruyere, DE Horsman, P Tsang, H Leitch, and A Karsan. (2010). Array comparative genomic hybridization of peripheral blood granulocytes of patients with myelodysplastic syndrome detects karyotypic abnormalities. American Journal of Clinical Pathology, 134(1):119-126. PMID: 20551276
14. **Starczynowski DT**, S Vercauteren, S Sung, A Brooks-Wilson, J Spinelli, C Eaves, A Eaves, D Horsman, W Lam, and A Karsan. (2011). Copy number alterations at polymorphic loci may be acquired somatically in patients with myelodysplastic syndromes. Leukemia Research, 35(4): 444-447. PMID: 20801506
15. **Starczynowski DT**, RD Morin, A McPherson, J Lam, R Chari, J Wegrzyn, A Delaney, AL Prabhu, Y Zhao, M Hirst, W Lam, MA Marra, and A Karsan. (2011). Genome-wide identification of human microRNAs located in leukemia-associated genomic alterations. Blood, 117(2): 595-607. PMID: 20962326
16. **Starczynowski DT**, F Kuchenbauer, J Wegrzyn, K Humphries, and A Karsan. (2011). MicroRNA-146a disrupts hematopoietic differentiation and survival. Experimental Hematology, 39(2): 167-178. PMID: 20933052
17. Kuchenbauer F, SM Mah, M Heuser, A McPherson, J Ruschmann, A Rouhi, T Berg, L Bullinger, B Argiropoulos, RD Morin, D. Lai, **DT Starczynowski**, A Karsan, CJ Eaves, A Watahiki, Y Wang, SA Aparicio, A Ganser, J Krauter, H Doehner, K Doehner, MA Marra, FD Carmargo L Palmquist, C Buske, and RK Humphries. (2011). Comprehensive analysis of mammalian miRNA* species and their role in myeloid cells. Blood, 118(12) 3350-3358. PMID: 21628414
18. Rhyasen G and **DT Starczynowski***. (2011). Deregulation of microRNAs in Myelodysplastic Syndromes. Leukemia, 26(1): 13-22. PMID: 21852786
19. **Starczynowski DT**, WL Lockwood, S Delehouzee, R Chari, J Wegrzyn, M Fuller, MS Tsao, S Lam, AF Gazdar, WL Lam, and A Karsan. TRAF6 is an amplified oncogene bridging the Ras and nuclear factor-kB cascade in lung cancer. (2011). Journal of Clinical Investigation, 121(10)4095-4105. PMID: 21911935.
20. Vercauteren S, **DT Starczynowski**, S Sung, K McNeil, C Salski, C-L Jensen, W Lam, A Karsan. (2012). T

cells of patients with Myelodysplastic syndrome are frequently derived from the malignant clone. British Journal of Haematology, 156(3): 409-412.

21. Fang J, M Varney, and **DT Starczynowski***. Implication of miRNAs in the pathogenesis of MDS. (2012) Current Pharmaceutical Design, 18(22): 3170-3779. PMID: 22685174
22. Fang J, G Rhyasen, L Bolanos, C Rasch, M Varney, M Wunderlich, S Goyama, G Jansen, J Cloos, C Rigolino, A Cortelezzi, JC Mulloy, EN Oliva, M Cuzzola, **DT Starczynowski***. (2012). Cytotoxic effects of Bortezomib in MDS/AML depend on autophagy-mediated lysosomal degradation of TRAF6 and repression of PSMA1. Blood. 120(4): 858-867. PMID: 22685174
23. Guo F, S Zhang, M Grogg, JA Cancelas, ME Varney, **DT Starczynowski**, W Du, J Yang, W Liu, G Thomas, S Kozma, Q Pang, Yi Zheng. Mouse gene targeting reveals an essential role of mTOR in hematopoietic stem cell engraftment and hematopoiesis. (2013). Haematologica, in press. PMID: 23716557.
24. Rhyasen G, L Bolanos, J Fang, C Rasch, A Jerez, M Varney, M Wunderlich, C Rigolino, L Mathews, M Ferrer, N Southall, R Guha, J Keller, C Thomas, L Beverly, A Cortelezzi, EN Oliva, M Cuzzola, J Maciejewski, JC Mulloy, **DT Starczynowski***. Targeting IRAK1 as a novel therapeutic approach for Myelodysplastic Syndrome. (2013). Cancer Cell. 24(1): 90-104.
25. Fang J, B Barker, L Bolanos, A Jerez, H Makishima, K Komurov, HL Grimes, J Cancelas, JP Maciejewski, **DT Starczynowski***. An intrachromosomal gene network drives high-risk del(5q) myeloid malignancies. (2013). Submitted.

*Corresponding author

Invited Talks

UCLA, Stem Cell Program. (April, 2013). Seminar series/Invited talk

Myeloid Workshop, American Society of Hematology, Atlanta, GA. (Dec, 2012). Invited talk.

James Graham Brown Cancer Center, University of Louisville, Louisville, KY. (Oct, 2012). Seminar series/Invited talk

Hematologic Malignancies, Houston, TX (October, 2012). Invited talk.

Midwest Blood Club Symposium, Indianapolis, IN (March, 2012). Oral Abstract.

American Society of Hematology, San Diego, CA (December, 2011): New Therapies in Myelodysplastic Syndromes. Oral Abstract Session.

MD Anderson, Leukemia Group (November, 2011)

Japanese Society of Hematology, Nagoya, Japan (October, 2011). Plenary Session.

Baltic Stem Cell Meeting, Szczecin, Poland (May, 2011). Plenary Session.

Midwest Blood Club Symposium, Cincinnati, OH (April, 2011). Plenary Session.

Cepheid Inc., Sunnyvale, CA (March, 2011).

Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH (March, 2011). Translational Hematology and Oncology Research Lecture Series.

Ulm University, Ulm, Germany (February, 2011). Workshop on Translational Research: Cellular and Molecular Biology of Cancer.

James Graham Brown Cancer Center, University of Louisville, Louisville, KY (February, 2011).

Keystone Symposia, NF- κ B in Inflammation and Disease, Santa Fe, NM (January 2010). Workshop: NF- κ B in Disease Pathogenesis.

Department of Cancer and Cell Biology, University of Cincinnati, OH (November, 2009). Department Seminar Series.

Department of Cytogenetics, Vancouver Hospital and Health Sciences Centre, Vancouver General Hospital, Vancouver, BC (March 2009). Cytogenetics Department Seminar Series.

Center for Advanced Biotechnology and Medicine, Rutgers University, New Brunswick, NJ (February 2009). Seminar Series.

Centre for Blood Research, University of British Columbia, Vancouver, BC (February 2009).
Centre for Blood Research Seminar Series.

American Society of Hematology, San Francisco, CA (December 2008). Myelodysplastic Syndromes: Basic Biology: Ribosomal Proteins, MicroRNAs and Animal Models. Oral Abstract Session.

Stem Cell Network, Annual General Meeting. Vancouver, BC (November 2008). Plenary Session.

BC Cancer Agency Annual Conference, Vancouver, BC (November 2008). Knowledge Translation - Basic vs. Clinical Research: Hematological Malignancies.

International Society of Experimental Hematology. Boston, MA. (July 2008). New Investigator Session.

Editorial Board

2012- Leukemia

2012- PLoS One

Ad Hoc Reviewer

PLoS One, Genes and Cancer, Current Pharmaceutical Review, Experimental Hematology, Leukemia, Blood, International Journal of Cancer, Haematologica, Journal of Hematology & Oncology, Annals of Hematology, Leukemia & Lymphoma, Nature Communication.

Mentoring

1. Garrett Rhyasen – PhD student; Department of Cancer and Cell Biology, University of Cincinnati (June, 2011 – current)

2. Jing Fang - Postdoctoral fellow; Cincinnati Children's Hospital Medical Center
(March, 2011 – current)
3. Melinda Varney – Postdoctoral fellow; Cincinnati Children's Hospital Medical Center
(February, 2011 – current)
4. David Miller (BSc candidate): Undergraduate; Xavier University
(June, 2012 – December, 2012)
5. Christopher Rasch – technician: Cincinnati Children's Hospital Medical Center
(August, 2011 – October, 2012)
6. Lyndsey Bolanos – lab manager/technician: Cincinnati Children's Hospital Medical Center
(February 2011 – current)
7. Brenden Barker – technician: Cincinnati Children's Hospital Medical Center
(July 2012 – current)